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Authors

Wong, Lian
Pegan, Jonathan D
Gabela-Zuniga, Basia
et al.

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NOTE

Leaf-inspired microcontact printing vascular patterns

Lian Wong[1,2], Jonathan D Pegan[3], Basia Gabela-Zuniga[1], Michelle Khine[3] and Kara E McCloskey[1,2]

[1] School of Engineering, University of California, Merced, United States of America
[2] Graduate Program in Biological Engineering and Small-scale Technologies, University of California, Merced, United States of America
[3] Department of Biomedical Engineering, University of California, Irvine, United States of America

E-mail: kmccloskey@ucmerced.edu

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Abstract

The vascularization of tissue grafts is critical for maintaining viability of the cells within a transplanted graft. A number of strategies are currently being investigated including very promising microfluidics systems. Here, we explored the potential for generating a vasculature-patterned endothelial cells that could be integrated into distinct layers between sheets of primary cells. Bioinspired from the leaf veins, we generated a reverse mold with a fractal vascular-branching pattern that models the unique spatial arrangement over multiple length scales that precisely mimic branching vasculature. By coating the reverse mold with 50 μg ml^{-1} of fibronectin and stamping enabled selective adhesion of the human umbilical vein endothelial cells (HUVECs) to the patterned adhesive matrix, we show that a vascular-branching pattern can be transferred by microcontact printing. Moreover, this pattern can be maintained and transferred to a 3D hydrogel matrix and remains stable for up to 4 d. After 4 d, HUVECs can be observed migrating and sprouting into Matrigel. These printed vascular branching patterns, especially after transfer to 3D hydrogels, provide a viable alternative strategy to the prevascularization of complex tissues.

Introduction

A major obstacle in the development of tissue engineered products for clinical applications is the lack of perfusable prevascularization within the *in vitro*-generated tissue product. This is especially important for building large organs such as the heart, kidneys and liver. Current strategies for *in vitro* prevascularization include: subtractive methods such as stainless steel needle-based molding and dissolvable-network-based sacrificial molding, additive methods such as soft lithography/PDMS stamping-based micromolding and layer-by-layer stacking, and hybrid methods such as bioprinting and gel-based microfluidic systems [1]. These various vasculogenesis and angiogenesis-based strategies also utilize a number of techniques (reviewed in [2]) including: photolithography, microcontact printing, functionalization of scaffold material, growth factor gradients and co-culture with mural cells [3, 4]. Specifically, microfluidic systems [5–7] have emerged as leading tools to overcome many of the challenges of developing microvasculature.

Although, microfluidic platforms offer precise control over various aspects of the cellular microenvironment enabling the generation of perfusable microvessels, the utility of these platforms remain limited. These platforms are limited to one length scale and are not currently designed to integrate the perfusable vasculature generated within the device with the primary tissue product. Moreover, it is becoming increasingly apparent that endothelial cells (ECs) provided with a tissue co-culture are highly migratory and disruptive to the patterned tissue-specific cells [8]. Tissue co-cultures with ECs have been successfully used to generate functional *in vitro* models in the blood-brain barrier [9], skin [10] and the lung alveolar capillary barrier [11]. A remaining challenge in these co-culture models is the generation of a basement membrane for physically separating the EC from the tissue-specific cells.

Alternatively, decellularized matrix from heart [12], liver [13] and kidney [14] are especially promising strategies for organ replacement because these strategies preserve the native three-dimensional (3D)

architecture and vascularity of the organ. However, the cell seeding often requires long culture times and tissue morphology and/or organization is difficult to reestablish [12]. Moreover, a failure to completely decellularize a tissue can lead to negative outcomes upon *in vivo* implantation, including a pro-inflammatory response with associated M1 macrophages and subsequent fibrosis. The complete endothelization of the entire vasculature is also a critical aspect for the success of a decellularized graft. Unless the graft is fully endothelialized to conceal the underlying collagen, coagulation and blockage will occur when the graft is exposed to circulating blood [13].

One potential alternative for providing semi-structured vasculature is to transfer patterned EC as distinct layers between sheets of cell/tissues or on the surface of biomaterials. Microcontact printing proteins is a well-known technique that is utilized to control spatial patterning and cell–cell interactions [15]. However, the successful biomimetic replicate of a highly branched vascular tree requires the anatomical structure of the native vasculature include branching over various length scales. Analogous to the transport of oxygen and nutrients in the blood vessel, the leaf contains veins that transport food and water to the plant. The length scales in the leaf can also mimic our microcapillary system. The veins of the leaf can also branch into smaller and smaller tributaries, just like the vascular system. Specifically, net-veined or reticulate-veined leaves contain veins that branch from the main rib with subdivisions into finer veinlets, extending from a midrib to the edge (elm, peach, apple, cherry), or radiate fan-shaped (maple, grapes). Some leaves are even designed in a parallel configuration (tulip). These lessons from nature can be exploited to create innovative designs in building new tissues.

By generating a vascular fractal-like pattern reverse mold from the veins of a leaf, we were able to model the unique spatial arrangement of EC over several length scales that precisely mimic branching vasculature. Here, we show that this can be accomplished via (1) microcontact printing adhesive matrices in appropriate vascular-like patterns followed by (2) transfer of the patterned cells to a 3D gel. This pattern is maintained after transfer to the surface of a 3D hydrogel matrix. The unique design potential for this largely 2D pattern would be the ability to transfer patterned EC between layers of cell sheets, potentially providing a non-disruptive alternative to pre-vascularizing complex tissues.

Methods

Mask

The mask is prepared by boiling a fresh leaf from a White Alder (Alnus rhombifolia) in a 0.2 molar Na_2CO_3 solution for 2 h. After boiling, the excess cellulose material surrounding the leaf vascular structure is removed manually with a nylon brush, rinsed with dH_2O and sandwiched between cardboard panels to dry overnight on the benchtop.

Leaf mold

The SU-8 2050 mold is soft baked on a hotplate at 65 °C for 3 min and then 95 °C for 6 min. The leaf mask is pressed flat against the SU-8 exposed to ultraviolet (ABM UV, I-line) for 15 s. A post exposure bake is performed on a hotplate at 65 °C for 2 min and 95 °C for 6 min. The SU-8 mold is then developed in SU-8 Developer (Microchem) for 5 min, rinsed with isopropyl alcohol and air dried, then hard baked at 200 °C for 30 min (figure 1).

Vascular stamp

Polydimethylsiloxane (PDMS) prepolymer and a curing agent were thoroughly mixed in a 10:1 weight ratio (Sylgard 184 Silicon Elastomer Kit, Dow Corning) and degassed in a desiccator for 30 min to remove any air bubbles in the mixture. The prepolymer mixture was poured onto the leaf mold and set to cure at 60 °C for 2 h. The next day, the PDMS stamp was peeled off of the mold, cut to desired stamp size (figure 2) and sterilized following standard procedures.

Microcontact printing

The PDMS stamping process (figure 2) follows by coating (or 'inking') the stamp surface with 50 μg ml^{-1} of fibronectin (FN; Corning) and incubated at 37 °C. After 1 h, the excess FN is removed by rinsing with distilled water followed by air drying. The FN-coated stamp is then applied directly to the surface of a non-tissue culture-treated dish using slight pressure and left in contact with the dish for 5 min before removal. The plate is incubated with 2% Pluronic® F-127 (Sigma) in phosphate buffered saline (PBS) solution for 1 h before washing with PBS.

Validation of printed FN

In order to better visualize the stamped FN pattern, Alexa Fluor 488 succinimidyl ester (Life Technologies) was conjugated to FN (Corning) at a 10-fold molar excess of fluorophore-to-protein and developed on a nutation device for 90 min at room temperature. The protein-fluorophore mixture was then purified through a size-exclusion column (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane; Millipore) and centrifuged according to manufacturer's directions. The final concentration of the protein-fluorophore was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The 2.4 mg ml^{-1} solution was then diluted down to 50 μg ml^{-1} for coating the stamp.

Cell culture

Human umbilical vein endothelial cells (HUVECs; Life Technologies) were cultured in a humidified

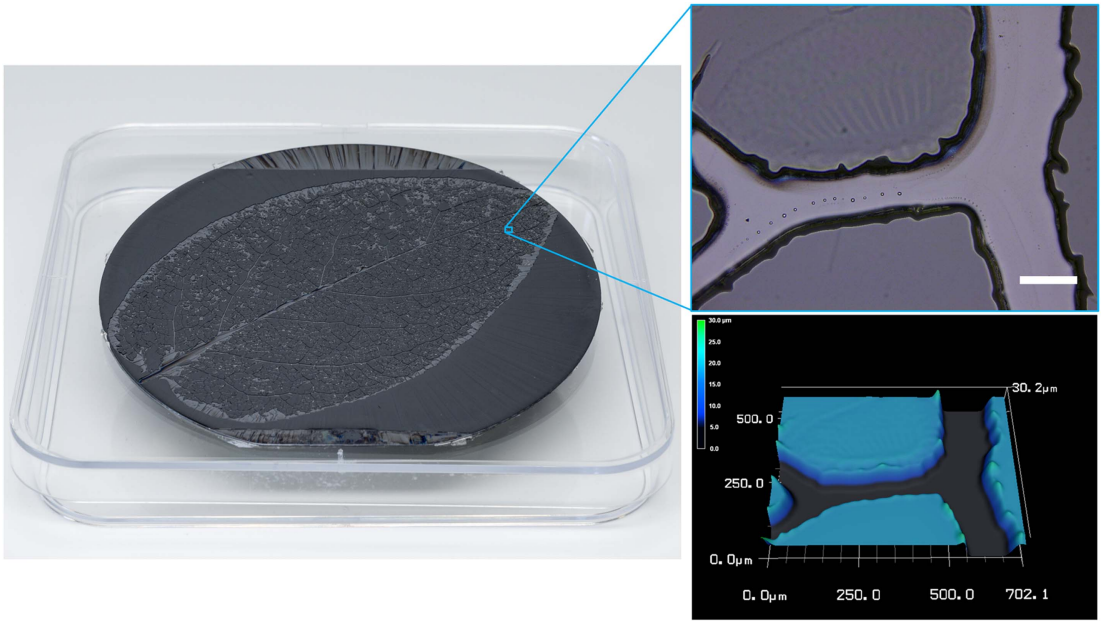

Figure 1. *Leaf mask*. After the mask is prepared by boiling a fresh leaf in a 0.2 molar Na2CO3 solution for 2 h and removing the excess cellulose material , the leaf is pressed into the surface of a SU-8 epoxy-based negative photoresist, treated with UV, baked and then developed. The resulting height profile of the patterned channels left in the mask measured ~25 μm in height. Scale bar = 100 μm.

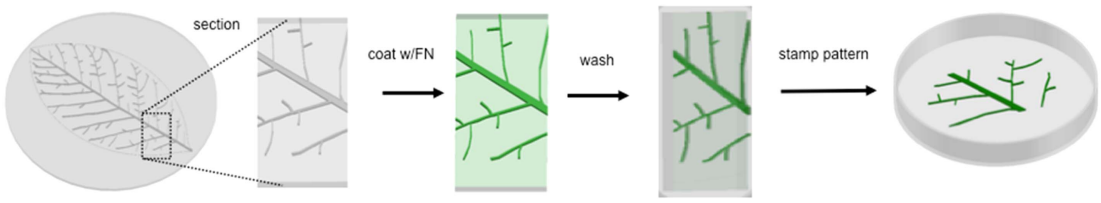

Figure 2. *Schematic of the stamping process*. A section of the PDMS leaf stamp is cut and coated with 50 ng ml^{-1} FN. After a gentle wash, the FN-coated stamp is pressed onto the surface of a non-tissue culture treated dish, thus transferring the FN pattern onto the surface of the dish.

incubator at 37 °C under 5% CO_2 in fully supplemented Endothelial Cell Growth Medium (EGM™-2 with BulletKit™; Lonza). HUVECs (p3-5) were then seeded onto the FN-patterned surface in a non-tissue culture-treated dish at 10 000 cell per cm^2 and imaged after 24 h.

Immunofluorescence staining and microscopy

The patterned HUVEC were fixed with 4% paraformaldehyde (PFA) for 20 min, permeabilized with 0.7% Triton X-100 for 5 min at room temperature, blocked for 20 min in 0.1% bovine serum albumin (BSA) in PBS and then incubated with Fluorescein Phalloidin (1:200; Life Technologies) for 1 h at room temperature. DAPI counterstain was added directly to the solution during the last 5 min of staining.

Collagen gels

Collagen gel solutions contained rat rail collagen type I (2 mg ml^{-1}; Corning), 0.1 M sodium hydroxide, 10% fetal bovine serum (Life Technologies), 20% 5× Dulbecco's Modified Eagle's medium (Life Technologies) and the remainder EGM-2 medium. The gel solution was then placed on patterned cells and cultured in a humidified incubator at 37 °C and 5% CO_2 overnight. After 24 h, the patterned cells have transferred from the stiffer cell culture dish to softer collagen gel surface. The collagen gel has also compacted to 80% of its original size and has detached from the dish.

Matrigel

Patterned HUVECs were coated Matrigel™ Basement Membrane Matrix at 50 μl cm^{-2} of growth surface and allowed to gel at 37 °C for 30 min. Culture media was then added and observed over time. After 4–7 d, the gel was rinsed gently with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After rinsing, phalloidin rhodamine (Invitrogen, 1:50) and DAPI (1:50) were added and allowed to stain for 24 h at 4 °C. Plates were rinsed twice before imaging using confocal microscopy (Nikon Eclipse C1).

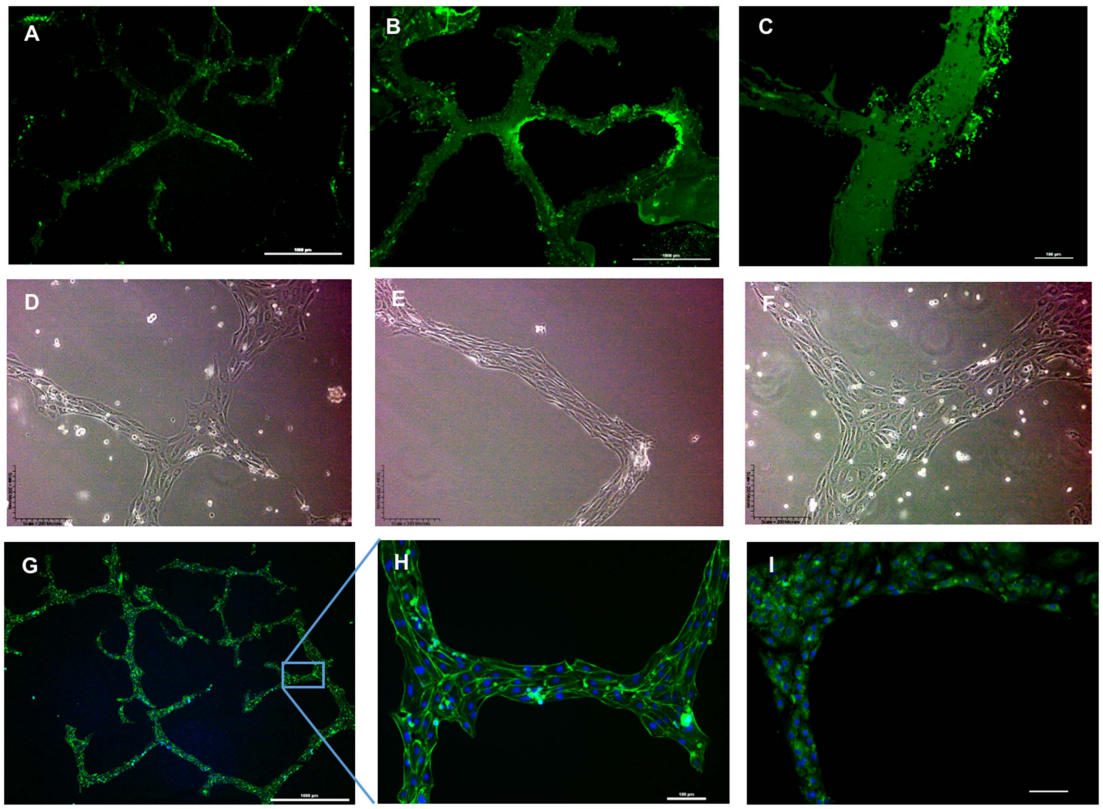

Figure 3. *Images of the patterned cells.* (A)–(C) In order to verify the selective stamping of the FN, the stamp was coated with FN-treated Alexa 488 (green). Scale bars = 1000 μm, 1000 μm and 100 μm, respectively. (D)–(F) After seeding with HUVECs, the cells adhere to the stamped vascular pattern, but not to the non-stamped areas in the non-tissue culture-treated dishes. Scale bar = 200 μm. (G)–(I) The patterned were HUVECs stained with F-actin (green) and DAPI (blue) counterstain. Scale bars = 1000 μm, 100 μm and 100 μm, respectively.

Results

Using a leaf branching pattern, we were able to generate a mask, reverse mold and stamp that retained the initial branch pattern (figure 1). This branched pattern was then used to preferentially surface-treat (i.e. stamp) Alexa Fluor 488 succinimidyl ester-treated FN onto non-tissue culture-treated dishes retaining the same pattern (figures 3(A)–(C)). Once HUVECs were seeded in the dish, they preferentially adhered only to the FN-stamped areas in the dish (figures 3(D)–(F)), and thus retained the vascular-like branching pattern of the leaf. To further illustrate the structure and alignment of the cells, HUVECs were stained with phalloidin and counterstained with DAPI nuclear stain (figures 3(G)–(I)).

Although a simple and elegant technique, microcontacting printing using bulk stamps like our vascular leaf stamp comes with resolution limits. The upper limit is inherently set at the diameter of the largest vein in the leaf, measured at 229 μm. The lower resolution limit in our PDMS stamp was 29 μm, respectively, and corresponded with stamped patterned vascular cells at 22 μm (figure S1 is available online at stacks.iop.org/BF/9/021001/mmedia). Although leaf veins were present at lower diameters, the PDMS stamp was not able to transfer the printed pattern at this smaller resolution. However, this value is very close to the normal length scales for microvasculature which typically range from 5 to 10 μm in diameter, and therefore, are expected to be sufficient for clinical application.

After the cells reached confluence on the pattern (day 3–4), they were transferred onto a 3D collagen-type I gel by direct cell-to-gel contact (figures 4(A), (B)). Upon subsequent culture on the collagen-type I hydrogels, the HUVECs maintain their vascular patterns on the 3D gel surface with minimal EC invasion into the 3D gel with a maximal depth of 46 μm (see figures 4(C), (D) and movie S2, available online at stacks.iop.org/BF/9/021001/mmedia) at 4 d. Interestingly, the maximal EC depths were observed at the nodes between branch points.

In order to investigate invasion potential of the patterned EC, the cells were also transferred to softer Matigel materials. Again, the patterned remained stable for up to 4 d. After 4 d, the EC began to migrate outside the pattern and invade into the Matrigel (figure 5).

Discussion

The vascular patterns developed using our microcontact printing methods enable patterning cells into

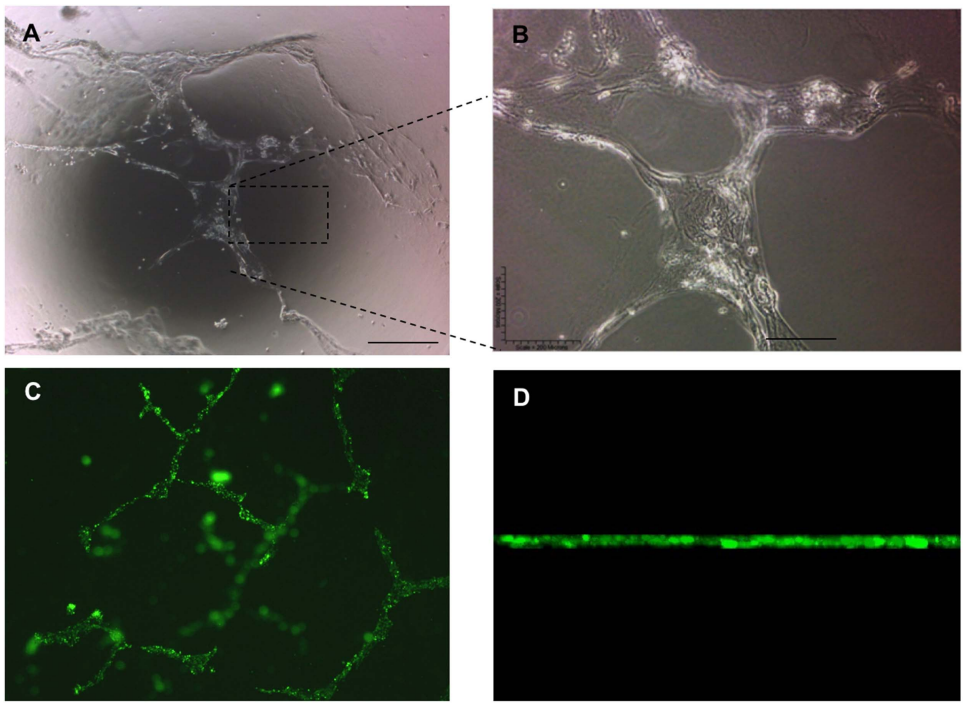

Figure 4. *Pattern transfer to collagen gels.* Patterned EC were transferred onto 3D collagen-type I gels by direct cell-to-gel contact (A) scale bar = 500 μm and (B) scale bar = 200 μm. Upon subsequent culture on the collagen-type I hydrogels, the HUVECs are able to (C) maintain their vascular patterns on the gel surface for up to 4 d (D) with minimal invasion into the 3D gel as seen by the Z-stacked image of the cells.

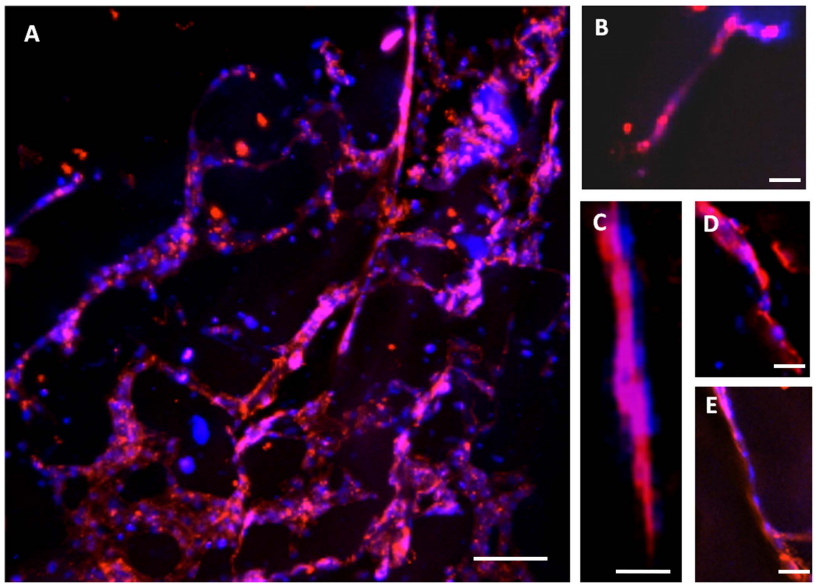

Figure 5. *Pattern transfer to Matrigel.* Patterned EC were transferred onto Matrigel by direct cell-to-gel contact. Individual cells can be seen by DAPI nuclear staining (blue) and phalloidin stain (red). Upon subsequent culture on the Matrigel, the HUVECs are able to maintain their vascular patterns on the gel surface for up to 4 d (not shown), but then (A) started to migrate (scale bar = 100 μm) and (B)–(E) invade the 3D gel (scale bar = 10 μm).

shapes mimicking the spatial scale of the microvasculature and the spatial organization of these cells is retained after transferring the cells from the stiffer tissue culture dish on to the surface of a softer 3D gel. This is an alternative design approach to perfusable microfluidic systems [5–7] in which the dynamic ECs are disruptive to the primary tissue assembly and organ function. In cardiac tissue, for example, the alignment of the cardiac cells facilitates the rapid cell-to-cell signaling required for synchronous contractile forces [16]. However, when dynamic ECs and neo vessels are co-cultured with patterned cardiac cell sheets, the migratory ECs disrupt the cell-to-cell junctions of the cardiac cells [8]. In these types of

in vitro cultures, dynamically sprouting neovessels, although perfusable, could disrupt the physiology of the primary tissue. Using the methods presented, patterned EC can be transferred onto 3D surfaces—potentially including cell/tissue/material grafts—enabling more rapid vascularization of implanted graft constructs and may, as recently shown from bioprinting 3D vascular channels with alternating primary tissue, facilitate a more robust vascularization of the transplanted graft *in vivo* [17].

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Conflicts of Interest

The authors declare no conflict of interest.

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